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(54) Title: METHOD OF LYOPHILIZATION AND RECONSTITUTION OF MIXTURES OF NUCLEATED NON-MAMMALIAN CELLS AND BLOOD MATTER

(57) Abstract

A process is disclosed for the lyophilization of a mixture of nucleated cells and blood matter which comprises the use of solutions comprising monosaccharide hexoses and pentoses, and a mixture of at least two biocompatible amphipathic polymers. The nucleated cells, such as cellular parasites, can be recovered in a viable state and the blood matter (erythrocytes, platelets and white blood cells) can be recovered in a viable state.

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METHOD OF LYOPHILIZATION AND RECONSTITUTION OF MIXTURES OF NUCLEATED NON-MAMMALIAN CELLS AND BLOOD MATTER

FIELD OF THE INVENTION

This invention relates to the general field of biochemistry and medical sciences, and specifically to novel lyophilized and reconstituted cell compositions comprising non-mammalian nucleated cells.

BACKGROUND OF THE INVENTION

Anaplasma marginale is a pathogen of considerable significance to the cattle industry. Anaplasmosis is often endemic in the tropics and subtropics, notably in the Americas and Africa, but also is prevalent in Australia, the South Pacific Islands, and southern Asia. In the USA it has been reported from all the contiguous states, but is most prevalent in the southeast, the intermountain west, and California. The rickettsiae are small, spherical bodies without cytoplasm and are located in the stroma or cytoplasm of the RBC. They range in diameter from 0.2-0.5μ and consist of an initial body that invades the red blood cell (RBC) and thereafter multiplies by binary fission within a vacuole to form inclusions consisting of 4-8 initial bodies that tend to be found toward the margin of the RBC, and may reach 1.0μ in diameter. These rickettsiae are therefore

obligate intracellular parasites, and require viable host red blood cells for infection.

Anaplasma centrale, which is more centrally located in the RBC, is a relatively nonpathogenic variant found in cattle in some regions; another variant known as A. ovis occurs in sheep and goats and may cause disease in stressful situations. Anaplasma spp infections also occur in a variety of wild ungulates such as deer and antelope, and these can act as reservoirs of infection for cattle.

One approach to dealing with these and other viral blood parasites, such as the feline parasite Toxoplasma, is to use vaccines of deactivated virus or viral particles. To develop such vaccines, infected blood samples are required and to ensure a continuing supply, the samples need to be stored for extended periods of time as lyophilates. A lyophilization and reconstitution procedure is thus needed which both conserves the useful characteristics of blood, which comprises non-nucleated cells and platelets, without killing the parasite, which comprises nucleated cells. It would therefore be desirable to devise a method for lyophilizing and reconstituting blood and nucleated cells, such as Anaplasma or Toxoplasma, so that the nucleated cells may be recovered in a viable state and the blood (erythrocytes, platelets and white blood cells) may be recovered in a useful state.

It is also desirable to obtain lyophilized nucleated non-mammalian cells, such as Anaplasma or Toxoplasma cells, along with their host red blood cells, which can be stored at high storage temperatures (4°C to room temperatures) with good shelf life.

The present invention provides a method for freeze-drying nucleated non-mammalian cells in the presence of red blood cells and platelets, in a manner which permits the reconstitution of the nucleated cells, as well as the red blood cells, platelets and white blood cells, with an intact cytoskeleton and with biologically-active hemoglobin, i.e., useful red blood cells. Useful RBC's can be characterized by one or more of the following: capability of synthesizing ATP; cell morphology; P_{50} values; oxyhemoglobin, methemoglobin and hemichrome values; MCV, MCH, and MCHC values; cell enzyme activity; and in vivo survival. When RBC's have been lyophilized according to previous methods, for example in either an aqueous or phosphate-buffered saline (PBS) solution, the reconstituted cells are damaged to the extent that the cells are not capable of metabolizing or synthesizing ATP, and the cell hemoglobin cannot transport oxygen. Damaged red blood cells cannot support obligate parasites such as Anaplasma, hence it is important to preserve viable, intact red blood cells when using infected cells as a vaccine.

SUMMARY OF THE INVENTION

The compositions provided by the present invention allow for storage of nucleated non-mammalian cells, particularly red blood cell parasites under normal conditions, while maintaining the structure and activity (viability) of the nucleated parasite cells and the host red blood cells. Briefly, the compositions are made by immersing a plurality of the nucleated non-mammalian cells and red blood cells (and platelets and white blood cells) in a physiologically buffered aqueous solution containing a carbohydrate, and a biologically compatible polymer, or mixture of polymers, preferably having amphipathic properties. By the term amphipathic it

is meant there are hydrophobic and hydrophilic portions on a single molecule. This immersion is followed by freezing the solution, and drying the frozen solution to yield novel freeze-dried cells containing less than 10%, and preferably about 3% or less by weight of moisture, which, when reconstituted, produce a significant percentage of viable, useful red blood cells and active nucleated cells. Methods of reconstitution of the cells are also provided.

DETAILED DESCRIPTION OF THE INVENTION

The carbohydrate utilized in the method according to the invention is biologically compatible with the cells, that is, non-disruptive to the cell membranes, and one which permeates, or is capable of permeating, the membrane of the cells. The carbohydrate may be selected from the group consisting of monosaccharides, since disaccharides do not appear to permeate the membrane to any significant extent. Monosaccharide pentoses and hexoses are preferred as is a final concentration of from about 7.0 to 37.5 weight % in phosphate buffered saline (PBS), preferably about 26%. Xylose, glucose, ribose, mannose and fructose are employed to particular advantage.

The invention will be hereafter described in connection with parasitic Anaplasma cells, as the nucleated non-mammalian cells, and red blood cells (host cells), but it will be understood it is also applicable to other types of nucleated cells, such as Toxoplasma.

It is particularly advantageous that the lyophilization and reconstitution procedures according to the present invention maintain viable

nucleated cells (the parasite) as well as viable host red blood cells which are critical for a viable parasite. The reconstituted parasite cells can then be used for preparation of vaccines after extended storage in a dry state. The reconstituted red blood cell and parasite mixture may also be injected directly as a vaccine formulation.

The polymer may be present in the solution in concentrations of from a final concentration of about 0.7 weight % up to saturation, and has a molecular weight in the range of from about 1K to about 600K. Preferably, the polymer has a molecular weight in the range of from about 2.5K to about 360K, most preferably from about 5K to 50K, and is present in a concentration of from about 3.6 weight % up to the limit of solubility of the polymer in the solution. Polymers selected from the group consisting of polyvinylpyrrolidone (PVP) and polyvinylpyrrolidone derivatives, and dextran and dextran derivatives provide significant advantages. Most preferred is the use of polyvinylpyrrolidone (an amphipathic polymer) of average molecular weight of in the range of 10-40K in an amount in the range of 12-20% weight to volume in the solution prior to lyophilization. Amino acid based polymers (i.e., proteins), dextrans or hydroxyethyl starch may also be employed. Other amphipathic polymers may be used, such as poloxamers in any of their various forms. The use of the carbohydrate-polymer solution in the lyophilization allows for the recovery of intact red blood cells, a significant percentage of which has normal morphologies, and exhibit viable cell metabolism as measured by synthesis of ATP. While not intending to be bound by any theory, the amphipathic properties of the polymer allow them to bind to the cell membrane while protecting the membrane surface by extension of

the hydrophilic portion into the aqueous environment. This may alleviate the damage to the cell membrane which causes other problems, such as cell aggregation.

The term lyophilization is broadly defined as freezing a substance and then reducing the concentration of the solvent, namely water, by sublimation and desorption, to levels which will no longer support biological or chemical reactions. Usually, the drying step is accomplished in a high vacuum. However, with respect to the storage of cells the extent of drying (the amount of residual moisture) is of critical importance in the ability of cells to withstand long-term storage at room temperature. In the method of the invention, cells may be lyophilized to a residual water content of less than 10 weight %, preferably less than 3%, and still be reconstituted to useful cells.

The lyophilization solution will be buffered in the range of pH of 7.0 to 7.4 preferably by a phosphate-buffered saline solution. A typical phosphate-buffered saline solution will comprise mono- and di-basic sodium phosphate (usually around 10 mM), sodium chloride (usually about 150 mM). This solution maintains the pH at around 7.2.

A preferred phosphate-buffered saline (PBS) solution to be used as the lyophilization buffer will comprise pyruvate, inosine, adenine, potassium chloride, sodium chloride, and dipotassium phosphate, all of which will serve as a basic salt buffer at a pH of about 7.2. In addition this lyophilization buffer will contain a final concentration of about 30% weight by volume of a monosaccharide, preferably 1.7 M glucose, and a final concentration of about 16%

weight by volume of a polymer, preferably polyvinylpyrrolidone (average molecular weight of 24K).

A mixture of polymers, preferably amphipathic polymers, may be used instead of a single polymer. The mixture of polymers may be present in the buffered lyophilization solution in total concentrations of from 0.7% (by weight) up to saturation. Preferably, each of the polymer types in the mixture has a molecular weight in the range of from about 1K to about 600K (number average molecular weight). Preferably, at least one of the types of polymers of the mixture will have a molecular weight from about 1K to 400K, and most preferably from 2.5K to 360K. Each of the polymer types may be present in a concentration of from about 3% (by weight) up to its limit of solubility in the buffered lyophilization solution. Also, one of the types of polymers of the mixture will have a molecular weight in the range of about 100K to about 600K, most preferably in the range of about 100-500K. Polymers selected from the group consisting of polyvinylpyrrolidone (PVP), polyvinylpyrrolidone derivatives, dextran, dextran derivatives, amino acid based polymers (i.e., proteins) and hydroxyethyl starch (HES) may be employed. Other amphipathic polymers may be used, such as poloxamers in any of their various forms. In a preferred embodiment, 3% PVP (molecular weight of about 360K) or 3% HES (molecular weight in the range of about 100K-500K) is employed in the buffered lyophilization solution.

The host red blood cells and the parasitic nucleated cells will preferably be prepared from whole blood centrifugation of blood containing the red blood cells infected with the parasite, mutation of the

parasite, or attenuated parasite strain, with the lyophilization buffer described above so that the final diluted concentration of carbohydrate and polymer are maintained in the necessary ranges.

Alternatively, packed red blood cell concentrates containing the parasite, parasite mutant or attenuated parasite strain may be used, prepared in CPDA (commercial solution containing citrate, phosphate, dextrose and adenine).

Upon lyophilization by conventional techniques to a moisture content of less than 10%, and preferably less than 3%, the lyophilized cells may be maintained under vacuum in vacuum-tight containers, or under nitrogen or other inert gas, at room temperatures for extended periods of time in absence of or without significant degradation of their desirable properties when reconstituted for use. It is a particular advantage of the present invention that the lyophilized cells may be stored at room temperature or refrigerated for extended periods of time.

It is a further advantage of the present invention that the lyophilized cells may be reconstituted at normal temperatures, i.e. greater than about 17°C up to about 37°C, which corresponds to normal human body temperature, and preferably at room temperature (about 22°C). The reconstitution medium is preferably a solution comprising a polymer or mixture of polymers having a molecular weight of from about 1K to 360 K, preferably 5K to about 360K, present in a concentration in the range of about 10 to 30% weight by volume. This polymer may be the same polymer utilized to lyophilize the red blood cells as described above. Hence the polymers polyvinylpyrrolidone, hydroxyethyl starch, and

dextran are particularly preferred and most preferred is polyvinylpyrrolidone present in a concentration of about 19% weight by volume in the reconstitution solution. The reconstitution solution will be buffered again typically by phosphate-buffered saline as described hereinabove to maintain a pH within the range of about 7.0 to 7.4. The most particularly preferred polymer is polyvinylpyrrolidone of an average molecular weight of about 10K.

The most preferred reconstitution buffer will be a solution comprising potassium chloride, sodium chloride and sodium phosphate and potassium dihydrogen phosphate, all of which form a basic salt buffer at a pH of about 7.2, which also contains about 19% weight by volume of polyvinylpyrrolidone (average molecular weight about 10K).

The reconstitution solution may also optionally contain a monosaccharide, preferably present in the concentration range of about 7.0 to 37.5% weight to volume. The preferred monosaccharides are xylose, glucose, ribose, mannose and fructose.

In the most preferred embodiment, the lyophilized cells can be reconstituted by mixing with an equal volume of the reconstitution buffer at a temperature of about 37°C and mixed until fully hydrated. By "equal" it is meant that the volume is the same as the starting volume prior to lyophilization. After reconstitution, the solution is preferably diluted 1:1 with dextrose-saline solution, at pH 7 and 290 mOsm.

Then, it is preferred that the rehydrated cells be washed according to the following procedure. It is realized, however, that once the cells are

reconstituted with reconstitution buffer they are in a useful form, but the combination of washings described hereinaftar are preferred, specifically to optimize retention of intact cells.

After separating the cells from the reconstitution buffer by centrifugation, the resulting packed cells, usually in the form of a pellet, are preferably resuspended in (approximately the volume used in the reconstitution) a buffer comprising the basic salt buffer at pH 7.2, described above, further containing about 10% weight by volume polyvinylpyrrolidone (molecular weight about 2.5K). Separation by centrifugation completes the first post-rehydration step, a washing step. The cells can be used as is or stored refigerated prior to injection.

The lyophilized nucleated cells and their host cells are advantageously storable at ambient atmospheric temperatures (i.e., room temperatures 20-30°C) and can be reconstituted to viable states. This allows for the preparation and storage of viable parasite cells or attenuated viable parasite cells which are useful to prepare vaccines to the parasite. Although vaccines comprising non-viable parasite cells or particles may also be useful, it is preferred that viable parasites be recovered since viable attenuated parasites in some instances are the preferred vaccine.

Having described the preferred embodiments of the present invention, the following examples are provided by way of illustration but are not intended to limit the invention in any way.

EXAMPLE I

Samples of bovine and sheep red blood cells, infected with *Anaplasma marginale* were washed with dextrose-saline to remove the buffy coats. Three different lyophilization buffer solutions were tried; at hematocrits of 10%, 30% and 40%:

1. 2.3 M Glucose + 2.5% C-30 PVP
2. 2.3 M Glucose + 3% 360K HES
3. 1.7 M Glucose + 3% 360K PVP + 1.5% HES

The lyophilization cycle patterns used were:

freeze - slow
drying cycle time - 6 days
sample size - 1.4 mls

Samples were reconstituted by mixing a volume equal to the original volume of the following reconstitution buffer with the dried material at 37°C.

Reconstitution Buffer

KCl	2.0mM	pH 7.20 ± 0.05
KH_2PO_4	1.47mM	605 ± 15 mOsm
NaCl	110.7mM	
Na_2HPO_4	8.1mM	
Plasdone C-15	190.0g/l	

This solution was swirled until all dry material was rehydrated. Samples were then stained and viable organisms were counted. A fluorescein diacetate-ethidium bromide viability staining procedure was used. Live organisms stain green and dead organisms stain orange. Numerous viable organisms were observed in their intact host red blood cells, indicating that a substantial amount of the *A. marginale* survived the lyophilization and reconstitution procedures.

From the foregoing description, one skilled in the art can readily ascertain that essential

characteristics of the invention and, without departing from the spirit and scope thereof, can adapt the invention to various usages and conditions. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient, and although specific terms have been employed herein, they are intended in a descriptive sense and not for purposes of limitation.

WHAT IS CLAIMED IS:

1. A process for the lyophilization of a mixture of nucleated cells and blood matter comprising:

immersing said mixture in a buffered solution which includes:

a monosaccharide which is present in the solution in a concentration of from about 7.0 to 37.5%, and

polymers having a number average molecular weight in the range of about 1K to about 600K, wherein the total concentration of said polymers is of from about 0.7% up to saturation in the solution; and

drying the cells by sublimation of the water.

2. The process of Claim 1 wherein said polymers are amphipathic.

3. The process of Claim 1 wherein said polymers comprise at least two types, one having a molecular weight in the range of about 20K to about 360K and another having a molecular weight in the range of about 100K to 500K.

4. The process of Claim 1 wherein the monosaccharide is selected from the group consisting of pentoses and hexoses.

5. The process of claim 4 wherein the monosaccharide is selected from the group consisting of xylose, glucose, ribose, mannose and fructose.

6. The process of Claim 3 wherein said mixture of polymers comprises polyvinylpyrrolidone and hydroxyethyl starch.

7. A process according to any of Claims 1 through 6 wherein said cells comprise erythrocytes.

8. A process according to any one of Claims 1 through 6 wherein said cells comprise platelets or white blood cells.

9. A process according to any one of Claims 1 through 6 wherein said nucleated cells are selected from the group consisting of cellular parasites, attenuated parasite strains, and fragments thereof.

10. A process according to Claim 9 wherein said parasites are viable.

11. A process according to Claim 10 wherein said parasite comprises Anaplasma.

12. A process according to claim 11 wherein said parasite comprises Anaplasma marginale.

13. A process of reconstituting a lyophilized composition of nucleated cells and blood matter comprising the step of:

mixing said composition with a sufficient volume of a phosphate-buffered saline reconstitution solution having a pH in the range of about 7.0-7.4 at a temperature in the range of about 15-50°C, said reconstitution solution comprising a final concentration of about 0.7% by weight up to the saturation concentration of a polymer having a molecular weight in the range of about 1K to 360K, to thereby reconstitute said nucleated cells to a useful state.

14. A process according to Claim 13 wherein said polymers are amphipathic.

15. A process according to Claim 13 wherein said polymers have a molecular weight in the range of about 2.5K to 360K.

16. A process according to Claim 13 wherein said nucleated cells are selected from the group consisting of cellular parasites, attenuated parasite strains, and fragments thereof.

17. A process according to Claim 16 wherein said parasites are viable.

18. A process according to Claim 17 wherein said parasite comprises Anaplasma.

19. A process according to Claim 18 wherein said parasite comprises Anaplasma marginale.

20. A process according to Claim 13, further comprising the steps of:

centrifuging said nucleated cells and blood matter and washing by at least one wash cycle by resuspending said cells in a dextrose-saline buffer solution at a pH in the range of about 7.0-7.4 and separating said cells from said buffer solution by centrifugation.

21. A process of reconstituting a lyophilized composition comprising nucleated cells and blood matter comprising the step of contacting said composition at a temperature greater than about 17°C with an aqueous solution of a polymer or mixture of polymers having a molecular weight of from about 1K to about 600K which is present in a final concentration in the range of 10 to 30% by weight.

22. A process according to Claim 21 wherein said polymers are amphipathic.

23. A process according to Claim 21 where said polymers have a molecular weight in the range of about 2.5K to 360K.

24. A process according to Claim 21, 22 or 23 wherein said polymer is selected from the group consisting of polyvinylpyrrolidone, hydroxyethyl starch, dextran and mixtures thereof.

25. A process according to Claim 22 wherein said polymer comprises polyvinylpyrrolidone of average molecular weight of about 10K.

26. A process according to Claim 21, 22 or 23 wherein said solution further comprises a monosaccharide in a final concentration of about 7.0 to 37.5% by weight.

27. A process according to Claim 21 wherein said nucleated cells are selected from the group consisting of cellular parasites, attenuated parasite strains and fragments thereof.

28. A process according to claim 27 wherein said parasites are viable.

29. A process according to Claim 28 wherein said parasite comprises Anaplasma.

30. A process according to Claim 29 wherein said parasite comprises Anaplasma marginale

31. A lyophilized composition comprising nucleated non-mammalian cells and host mammalian blood cells, said composition capable of storage at ambient atmospheric temperatures, and capable of reconstitution to restore said nucleated non-mammalian cells and said mammalian blood cells to viable states.

32. A composition according to Claim 31 wherein said blood cells comprise red blood cells.

33. A composition according to Claim 32 wherein said nucleated cells are selected from the group consisting of cellular parasites, attenuated parasite strains, and fragments thereof.

34. A composition according to Claim 33 wherein said nucleated cells comprise Anaplasma.

35. A composition according to Claim 34 wherein said nucleated cells comprise Anaplasma marginale.

36. A vaccine comprising a composition according to any one of Claims 31 through 35.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00650

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): IPC (5): A01N 1/02; C12N 5/00
US CL : 435/2, 240.1; 424/88

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/2, 240.1; 424/88

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

APS, Dialog files: 5, 155, 350

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	US, A, 4, 874, 690 (Goodrich et al) 17 October 1989, see entire document.	1-30
Y	Cryobiology, Volume 9, 1972, Ashwood-Smith et al, "Low-Temperature preservation of Mammalian Cells in Tissue Culture with polyvinylprrolidone (PVP), Dextrans, and Hydroxyethyl Starch (HES)", pages 441-449, see entire article.	21-26
Y	American Journal of Veternary Research, Volume 33, No. 12 issued December 1972, Love, "Crogenic Preservation of Anaplasma marginale with Dimethyl Sulfoxide", pages 2557-2560, see entire article.	29-36

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
13 MAY 1992	26 MAY 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	H. Sidberry

Form PCT/ISA/210 (second sheet)(May 1986) &

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

VI. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-12 and 31-36, drawn to a process for lyophilization of a mixture of cells, lyophilized composition and vaccine, Classified in Classes 435 and 424, Subclasses 2, 240.1 and 88.

II. Claims 13-20 are drawn to a process of reconstituting the lyophilized cells, Classified in Class 435, Subclasses 2 and 240.1.

III. Claims 21-30 are drawn to a second process of reconstituting the lyophilized cells, Classified in Class 435, Subclasses 2 and 240.1.

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